6 October 2011

Provisional Agenda

1 Opening of the meeting

2 Nomination of Chairperson, Vice-Chairperson, and Rapporteur

3 Adoption of the Provisional Agenda

4 General briefing on the working method of the meeting

5 Briefing on the background and preparation of updating the WHO/IUCN/WWF guidelines on conservation of medicinal plants (1993)

6 Introduction and perspectives of international organizations taking part in the update of the guidelines

- WHO
- IUCN
- WWF
- TRAFFIC
- FAO

7 Review and discussion of the outline of the draft updated guidelines

8 Review and discussion of the content of the draft updated guidelines

9 General discussion on the future process

10 Discussion on recommendations

11 Site visit to the relevant research stations in Toyama

12 Others, if any

13 Closure of the meeting

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CONTENTS

10 October 2011

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写真 1　原案提示

写真 2　修正事項討議

写真 3　集合写真

薬用植物を理解

県民公開講座

県民公開講座「意外と知らない身近な薬用植物」は15日、県民会館で開かれ、県民が漢方薬の原料となる薬用植物の現状や作用に理解を深めた。

富山大教員や県職員でつくる「薬用植物の保護に関するWHO会議実行委員会」（委員長・佐竹元吉富山大和漢医薬学総合研究所客員教授）が企画。世界保健機関本部の丸山紀子科学官、佐竹教授、同研究所の小松かつ子教授、門脇真教授の4人が講演した。

小松教授は、中国産の生薬は採集地の減少や富裕層の増加などで近年、価格が2～4倍に高騰しているとし、「国内で可能な薬用植物の栽培を推進し、栽培した薬用植物を流通させる仕組みをつくることが必要だ」と語った。

薬用植物の現状や作用について学んだ県民公開講座＝県民会館

記事 1 北日本新聞 2011 年 10 月 16 日

「薬用植物 保護に力」

WHO担当者ら 知事表敬訪問

きょうから富山 県民会館で専門家会議

15日から富山市の県民会館で薬用植物の保護に関する世界保健機関（WHO）の専門家会議が開かれるのに先立ち、張奇WHO本部伝統薬部門担当課長らが14日、石井隆一知事を表敬訪問した。【写真・記】

専門家会議は15～17日までで、欧州やアフリカなど世界各国から約60人の専門家が出席して「薬用植物の保護に関するガイドライン」の改訂について議論する。また、15日には会議に参加するWHOの科学者らによる県民公開講座が開かれる。

石井知事は張担当課長らに対し「薬用植物の保護に関する重要な会議が富山で開かれるのは誇りに思う」とあいさつ。また「富山県は全国で例がない県立の薬事研究所があり、薬用植物の研究や薬用植物の保護などに非常に力をいれている。今後も富山県と緊密な関係にしていきたい」と述べた。

石井知事（手前左端）と会談する張奇WHO伝統薬部門担当課長（奥右から2人目）＝県庁で

記事 2 北日本新聞 2011 年 10 月 15 日

薬用植物保護の指針改定へ

富山でWHO専門家会議

WHO専門家会議の開会式であいさつする張奇氏（中央）＝富山市のANAクラウンプラザホテル富山

薬用植物の保護に関するWHO（世界保健機関）専門家会議の開会式が14日、富山市のANAクラウンプラザホテル富山で開かれ、世界各国の専門家らがガイドライン改定を目指す会議の成功を誓い合った。

専門家会議は15日から17日まで、県民会館で開催。21カ国の専門家やWHO本部の職員ら約60人が参加し、WHO薬用植物伝統医薬部門担当課長が「富山県が1993年に策定した「薬用植物の保護に関するガイドライン」の改定版を取りまとめる。

開会式で、WHOの張奇伝統薬部門担当課長が「富山県は伝統医薬の推進と伝統薬の配置サービスで長い歴史と豊かな経験があり、県とWHOの継続的でより緊密な協力関係の進展を期待している」と述べ、石井知事は「会議の成果が世界の人々の健康増進につながるよう期待している」とあいさつ。田宮憲一厚生労働省医政局研究開発振興課長補佐と遠藤俊郎富山大学長も歓迎の言葉を述べた。

記事 3 毎日新聞 2011 年 10 月 15 日

薬用植物保護で結束

富山でWHO専門家会議開会

ガイドライン改訂へ協力

開会のあいさつをする張氏＝富山市内のホテル

薬用植物の保護に関するWHO）専門家会議の開催する世界保健機関（W会式は14日、ANAクラウンプラザホテル富山で行われ、20数カ国約60人の研究者らが薬用植物保護に関する新ガイドライン改訂へ協力、連携することを確認した。

WHO本部伝統薬部門担当課長の張奇氏が「富山県は伝統医学、家庭配置薬のサービスで長い伝統があり、この地で緊密的、協力的な進展を期待する」と会議の成功に意欲を示した。

石井隆一知事は各国からの出席者を歓迎し、「会議の成果が人類の健康増進につながること願う」と述べた。厚生労働省医政局研究開発振興課課長補佐の田宮憲一氏、遠藤俊郎富大学長があいさつした。

会議は15日から3日間にわたって富山市の県民会館で開かれるほか、15日に県民公開講座を開催、関係者は18日に富大民族薬物資料館などを見学する。

これに先立ち、WHO伝統薬部門の張奇課長らは県庁を訪れ、石井知事と懇談した。

県学生競書展

作品を募集

来月、南砺で開催

記事 5 富山新聞 2011 年 10 月 15 日

薬用植物の保護議論

富山 WHO専門家会議開幕

薬用植物の保護に関する世界保健機関（WHO）専門家会議が十四日、富山市内のホテルであり、五日間の日程で始まった。

WHOは「薬用植物の保護に関するガイドライン」の改訂を進めており、薬用植物の栽培促進や乱獲防止などで今回の議論を反映し、改訂指針を取りまとめる。二十一カ国の専門家六十人が十五～

会議の意義について語るWHO伝統薬部門の張氏＝県庁で

は七月の記者会見で「では」という議論の仕方もあると述べ、今後も粘り強く一部存続などを模索する考えを表明している。（相馬敬）

十七日、同市新総曲輪の県民会館で話し合う。十八日は県内の関連施設を見学する。

県民公開講座もあり、十五日午後一時半から県民会館でWHO本部科学官の丸山紀子氏ら四人が講演する。

WHO伝統薬部門の張奇課長が十四日、石井隆一知事を表敬し、「薬都の富山で会議を開催し、長い歴史と豊かな経験を共有することに重要性がある」と話した。（山田晃史）

知事と歓談するバッタ一二総領事＝県庁で

イタリアと富山の製薬業連携に意欲

県庁で駐大阪総領事

ディック（富山市）と、自国の製薬会社との交流促進に向けて来県した駐大阪イタリア総領事のマリオ・バッ

◇魚津産業フェア（ありそドーム）前11市職害者ふれあい交流フェスティバル（市障害者交流センター）

記事 4 北陸中日新聞 2011 年 10 月 15 日

薬用植物の保護に関するWHO会議

県民公開講座

意外と知らない身近な薬用植物

参加無料
事前予約不要

日時 平成23年 10月15日(土) 13:30～16:00

場所 富山県民会館401号室
(富山県富山市新総曲輪4-18)

講演

WHOを身近に－薬用植物を通じて
丸山由紀子(世界保健機関／WHO本部科学官)

身近な薬用植物
佐竹　元吉(富山大学和漢医薬学総合研究所・お茶の水女子大学教授)

漢方薬等の原料となる薬用植物の現状
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身近な薬用植物の意外な作用
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主催：薬用植物の保護に関するWHO会議実行委員会
後援：富山県、富山大学和漢医薬学総合研究所

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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻合	ページ	出版年
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RESEARCH ARTICLE

Chemical inducers of heat shock proteins derived from medicinal plants and cytoprotective genes response

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Abstract

Environmental stress induces damage that activates an adaptive response in any organism. The cellular stress response is based on the induction of cytoprotective proteins, the so-called stress or heat shock proteins (HSPs). HSPs are known to function as molecular chaperones which are involved in the therapeutic approach of many diseases. Therefore in the current study we searched nontoxic chaperone inducers in chemical compounds isolated from medicinal plants. Screening of 80 compounds for their Hsp70-inducing activity in human lymphoma U937 cells was performed by western blotting. Five compounds showed significant Hsp70 up-regulation among them shikonin was most potent. Shikonin was able to induce Hsp70 at 0.1 μM after 3 h without activation of heat shock transcription factor 1 (HSF-1). It also induces significant reactive oxygen species generation. The expression level of genes responsive to shikonin was studied using global-scale microarrays and computational gene expression analysis tools. Significant increase in the nuclear factor erythroid 2-related factor 2 (Nrf2, NFEL2L2) mediated oxidative stress response was observed that leads to the activation of HSP. The results of gene chip analysis were further confirmed by real-time qPCR assay. In short, the detailed mechanisms of Hsp70 induction by shikonin is not fully understood, Nrf2 and its target genes might be involved in the Hsp70 up-regulation of U937 cells.

Keywords: *Heat shock proteins, Nrf2, oxidative stress, shikonin*

Introduction

Human exposure to environmental toxicants has been associated with etiology of many diseases including inflammation, cancer, cardiovascular and neurodegenerative disorders. To counteract the detrimental effects of environmental insults, mammalian cells have evolved a hierarchy of sophisticated sensing and signalling mechanisms to turn on or off endogenous antioxidant responses accordingly [1]. The ability of cells to counteract stressful conditions, known as cellular adaptive response, requires the activation of pro-survival pathways and the production of molecules with antioxidant, antiapoptotic and proapoptotic activities [2]. Among the cellular pathways conferring protection against oxidative stress, a key role is played by vitagenes, which include heat shock proteins (HSPs) such as heme oxygenase-1 (HMOX1) and Hsp70, as well as thioredoxin/thioredoxin reductase system [3]. Heat shock or stress response is a cellular adaptive response, which contributes to establishing a cytoprotective state in a wide variety of human diseases. When appropriately

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activated, cellular stress response has the capability to restore cellular homeostasis and rebalance redox equilibrium [4].

Among the many changes in cellular activity and physiology, the most remarkable event in stressed cells is the production of a highly conserved set of proteins, the heat shock or stress proteins (HSPs) [5]. HSPs are also expressed constitutively at normal growth temperatures and play an essential role as molecular chaperones by assisting the correct folding of nascent and stressed accumulated misfolded proteins, preventing their aggregation [6], assembly/disassembly of multi-subunit oligomers, translocation of proteins across intracellular membranes, process of endocytosis, regulation of apoptosis and cytoskeletal organisation [7]. A number of studies have reported that molecular chaperones can confer cellular and tissue stress tolerance and provide beneficial effects on various pathological states, such as stress ulcers and ischaemia-induced injuries, as well as on diseases associated with protein misfolding and aggregation [8–10].

Given the broad cytoprotective properties of the heat shock response, there is now strong interest in discovering and developing pharmacological agents capable of inducing stress responses. Therefore, in the current study we screened some phyto-medicinal compounds for their HSP-inducing activity.

Materials and methods

Chemical compounds

Chemical compounds used in this study were obtained from the Institute of Natural Medicine, University of Toyama, Japan.

Cell culture

A human lymphoma cell line, U937 was obtained from the Human Sciences Research Resource Bank (Japan Human Sciences Foundation, Tokyo, Japan). The cells were maintained in Roswell Park Memorial Intitute (RPMI) 1640 medium (Sigma, St Louis, MO, USA) with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences Lenexa, KS, USA) and incubated in a CO_2 incubator with 5% CO_2 and 95% air at 37°C.

Hyperthermia treatment

Hyperthermia (HT) treatment was used as a positive control for induction of HSPs. Cells were collected and suspended in 2 mL fresh medium in plastic culture tubes, and were exposed to 44°C (±0.05°C) for 15 min in a water bath (NTT-1200, Eyela, Tokyo, Japan). After HT treatment, cells were incubated for desired time at 37°C in humidified air with 5% CO_2.

Assessment of apoptosis

For the detection of early apoptosis and secondary necrosis, Annexin V-FITC kit, purchased from Immunotech (Marseille, France), was utilised according to the manufacturer's recommendations. Briefly, cells were stained simultaneously with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labelled annexin V and assessed with a flow cytometer (Beckman-Coulter EPICS XL^{TM}).

Determination of cell survival by WST-8 assay

Cell survival was determined using a Cell Counting Kit-8. The cells (2×10^4 /well) in 96-well plates were incubated with various concentrations of shikonin for 6 h. After incubation, WST-8 reagents were added to each well. Absorbance at 450 nm was measured using a microplate reader (BioRad, Hercules, CA) after 2 h of incubation with WST-8 reagents. Absorbance is proportionally related to the number of live cells.

Western blot analyses for proteins

Western blot analysis was performed for Hsp70, HSF-1, NRF2 and β-actin by using specific polyclonal or monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as described in previous papers [11, 12]. For the detection of these specific antibodies the chemiluminescence ECL detection reagents were used following the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK).

RNA isolation

Total RNA was extracted from cells using an RNAeasy Total RNA Extraction kit (Qiagen, Valencia, CA) and treated with Dnase I (RNase-free Dnase kit, Qiagen) for 15 min at room temperature to remove genomic DNA.

Assessment of intracellular reactive oxygen species (ROS)

To measure intracellular superoxide (O_2^-) we used 2 μM hydroethidine (HE) (Molecular Probes, Eugene, OR) a dye that is oxidized within the cell and fluoresces when it intercalates into DNA. To measure intracellular peroxides including H_2O_2 5 μM dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) was utilised. DCFH-DA upon entering into the cells de-esterified and then oxidized to dichlorofluorescein (DCF). The fluorescence emission was analysed by flow cytometry [13].

High-density oligonucleotide microarray and computational gene expression analysis

Gene expression was analysed using a GeneChip® system with a Human Genome U133-plus 2.0 array (Affymetrix, Santa Clara, CA) spotted with 54,675 probe sets, more than twice the molecular probes used in our previous studies [14, 15]. Samples for array hybridisation were prepared as described in the Affymetrix GeneChip® Expression Technical Manual. Briefly, 5 g of total RNA was used to synthesise double-stranded cDNA with a GeneChip® Expression 30-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix). Biotin-labelled cRNA was then synthesised from the cDNA using GeneChip® Expression 3′-Amplification Reagents for IVT labelling (Affymetrix). After fragmentation, the biotinylated cRNA was hybridised to the GeneChip array at 45°C for 16 h. The arrays were washed, stained with streptavidin-phycoerythrin, and scanned using a probe array scanner. The scanned chip was analysed using GeneChip Analysis Suite Software (Affymetrix). The obtained hybridisation intensity data were converted into a presence or an absence call for each gene, and changes in gene expression level between experiments were detected by comparative analysis. The data were further analysed using Gene-Spring software (Silicon Genetics, Redwood City, CA) to extract the significant genes [16, 17]. To examine gene ontology, including biological processes, cellular components, molecular functions, and genetic networks, the obtained data were analysed using Ingenuity Pathways Analysis tools (Ingenuity Systems, Mountain View, CA), a web-delivered application that enables the identification, visualisation and exploration of molecular interaction networks in gene expression data. The gene lists identified by GeneSpring containing Affymetrix gene ID and the natural logarithm of normalised expression signal ratios from GeneChip CEL files were uploaded into the Ingenuity Pathways Analysis system. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These so-called focus genes were then used as a starting point for generating biological networks [16, 17].

Real-time quantitative PCR assay

Real-time quantitative PCR (qPCR) assay was performed on a real-time PCR system (Mx3000P, Stratagene, Tokyo, Japan) using SYBR PreMix ExTaq (Takara Bio, Shiga, Japan) or Premix ExTaq (for the use of TaqMan probes; Takara Bio) in accordance with the manufacturer's protocols. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, Qiagen) was carried out with DNase-treated total RNA using an oligo (dT) 16 primer. Real-time qPCR assay was performed using the specific primers listed in Table I. Each mRNA expression level was normalized to the mRNA expression level of GAPDH.

Results

Screening of medicinal compounds for Hsp70 up-regulation

Eighty medicinal compounds shown in Table 1 were examined for their ability to induce Hsp70 up-regulation. The screening procedure was accomplished by treating U937 cells with the compounds 1 to 10 at 0.1 mM for 24 h followed by western blot analysis. Among the tested samples no band of Hsp70 was observed with compounds 3–5 suggesting the toxicity of the dose (data not shown). Therefore, we selected compounds 3–5 and did concentration-dependent screening for Hsp70 up-regulation. U937 cells were treated with 0.1 μM, 1 μM and 10 μM for

Table I. List of 80 chemical compounds that were examined for their Hsp70 inducing ability.

1. Aconitine	17. Bufotalin	33. Epihesperidin	49. Gomisin N	65. Paeoniflorin
2. Albiflorin	18. Capillarisin	34. Ergosterol	50. Hesperidin	66. Paeonol
3. Alisol A	19. Capsaicin	35. beta-Eudesmol	51. Hirsutine	67. Palmatine chloride
4. Alisol B	20. Catalpol	36. (E)-Ferulic acid	52. Honokiol	68. (S)-Perillaldehyde
5. Alkannin	21. (E)-Cinnamic acid	37. Geniposide	53. Hypaconitine	69. Puerarin
6. Amygdalin	22. Cinobufagin	38. Geniposidic acid	54. Icariin	70. Rhynchophylline
7. Arbutin	23. Cinobufotalin	39. Gentiopicroside	55. Isofraxidine	71. Saikosaponin a
8. Astragaloside IV	24. Coptisine chloride	40. [6]-Gingerol	56. (Z)-Ligustilide	72. Saikosaponin b2
9. Atractylenolide III	25. Corydaline	41. Ginsenoside Rb1	57. Limonin	73. Saikosaponin c
10. Aucubin	26. Curcumin	42. Ginsenoside Rc	58. Loganin	74. Schizandrin
11. Baicalein	27. Dehydrocorydaline nitrate	43. Ginsenoside Rd	59. Magnolol	75. Sennoside A
12. Baicalin	28. Dehydrocostuslactone	44. Ginsenoside Re	60. Mesaconitine	76. Shikonin
13. Barbaloin	29. Dihydrocapsaicin	45. Ginsenoside Rg1	61. Naringin	77. [6]-Shogaol
14. Berberine chloride	30. Dimethylesculetin	46. Glabridin	62. Nodakenin	78. Sinomenine
15. Bergenin	31. Eleutheroside B	47. Glycyrrhizic acid	63. Osthole	79. Swertiamarin
16. Bufalin	32. (-)-Epigallocatechin gallate	48. Gomisin A	64. Oxymatrine	80. Wogonin

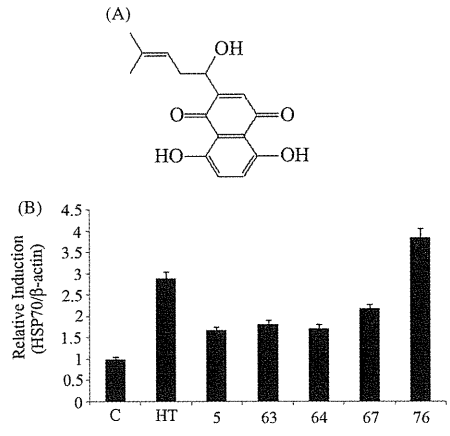

Figure 1. (A) Chemical structure of shikonin. (B) Bands of Hsp70 induced by five active compounds at 1μM concentration were quantified by densitometry and normalised with β-actin. Hyperthermia (HT) 44°C for 15 min was used as a positive control. Bars indicate standard deviation ($n=3$).

24 h followed by western blot analysis. Significant Hsp70 up-regulation was observed with compound 5 at 1 μM (densitometric ratio: control: 1.0 ± 0.08, 0.1 μM: 1.25 ± 0.12, 1 μM: 1.50 ± 0.14, 10 μM: 1.12 ± 0.13, mean ± SD, $n=3$) while compounds 3 and 4 did not show any significant increase at all concentrations as compared to control. From these results we selected 1 μM concentration for the screening of medicinal compounds.

Out of the 80 compounds, five (5, 63, 64, 67 and 76) showed significant Hsp70 up-regulation. Among them compound 76 (shikonin) was the most potent (Figure 1A, 1B).

Effects of shikonin on apoptosis induction in U937 cells

To determine the non-toxic concentration of shikonin, cells were exposed to the drug concentration-dependently for 6 h. This was followed by measurement of early apoptosis and secondary necrosis by annexin V FITC/PI staining using flow cytometry. No apoptosis was observed at 0.01 μM and 0.1 μM, significant apoptosis was observed at 1 μM concentration (Figure 2A).

Effects of shikonin on cell survival

To further confirm the non-toxic concentration, cell survival assay was performed with 0.01, 0.1 and 1 μM concentrations after 6 h incubation. The results also showed the toxicity at 1 μM while no toxicity was observed with 0.01 and 0.1 μM concentrations. (Figure 2B).

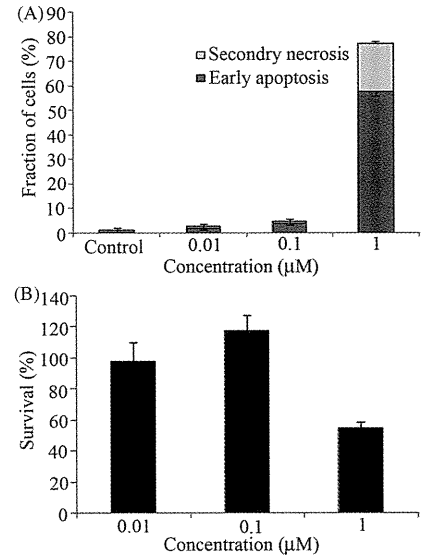

Figure 2. (A) U937 cells were treated with shikonin concentration-dependently for 6 h followed by assessment of early apoptosis (black bar) and secondary necrosis (grey bar) by flow cytometry. Bars indicate standard deviation ($n=3$). (B) Cell survival assay was performed with 0.01, 0.1 and 1 μM concentrations of shikonin. After 6 h incubation WST-8 reagent was added to each well. Bars indicate standard deviation ($n=3$).

Effects of shikonin on ROS formation

Previously it has been reported that shikonin can induce ROS formation [18]. Therefore, in the current study we investigated that whether at non-toxic concentration shikonin can induce ROS. Cells were exposed to 0.1 μM shikonin and the levels of DCF and HE fluorescence were monitored time dependently via flow cytometry. Intracellular peroxide level was increased as early as 30 min after treatment (Figure 3A), while no intracellular O_2^- was observed (data not shown).

Time-dependent effects of shikonin on Hsp70 induction

U937 cells were treated with 0.01 μM and 0.1 μM shikonin time-dependently, followed by western blot analysis. Up-regulation of Hsp70 was observed with 0.1 μM after 3 h incubation and continued to increase time-dependently, while no up-regulation of Hsp70 was observed at 0.01 μM shikonin (Figure 3B, 3C)

The transcription of *HSP* genes is regulated by transcription factor HSF, which senses cellular exposure to stress and turns on rapid induction of HSPs [19]. Therefore we examined the effects of shikonin on the activation of HSF1. U937 cells were